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13. ABSTRACT ***(Maximum 200 Words)***

Breast cancer metastases account for a significant morbidity and mortality in women. Efficient means of treating this subset of patients remain elusive. The purpose of this study is to determine whether intravenous (IV) injection of human Interleukin-2 gene-modified endothelial cells (hIL-2/MECs) can abrogate breast cancer metastases and prolonged survival of the tumor-bearing animals. Following the establishments of breast cancer metastases in the lungs of BALB/c mice, three doses of 10^5 hIL-2/MECs, spaced 72-h apart, were administered to the animals via the tail vein. Subsequently, the lungs of the mice were removed and examined for the presence and number of tumor foci following their death or sacrifice.

Lungs of untreated tumor-bearing mice contained numerous tumor foci, and they all died by day 23. Their average survival time was 21 days. In contrast, the lungs of hIL-2/MEC- treated tumor-bearing mice contained an average of fifty tumor foci per mouse. Their average survival time of 40 days. Animals which received only the hIL-2/MECs without tumor cells did not develop tumors and remained alive and well. These results suggest that IV administered, interleukin-2 gene-modified endothelial cells can inhibit the growth of established metastases of breast cancer and prolong the survival of tumor-bearing mice.

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1. INTRODUCTION

Breast cancer is the most common female malignancy in North America (1). This disease is estimated to affect 1 of 9 women (2), and is associated with substantial morbidity and mortality (1). While early detection and treatment have led to significant improvements in cancer -related mortality and quality of life for women with breast cancer, recurrence and metastatic dissemination of the tumors still account for a significant morbidity and mortality in patients. Effective means of treating this subset of patients remains elusive. A novel approach to the problem of recurrent or metastatic cancer involves the activation of potent immune responses that are capable of specifically destroying tumor cells. Transgenic immunotherapy, as the term implies, refers to the insertion of cytokine genes into cells in order to activate anti-tumor immune responses. Moreover, this approach is intended to avoid the dose-limiting toxicities that have impeded the application of otherwise very promising cytokine therapies. The goal of this research is to develop an effective and safe gene therapy for invasive breast cancer. The objectives of the research are (1) to determine whether intravenously (IV) administered endothelial cells expressing exogenous cytokine gene(s) can selectively migrate into pulmonary metastases of breast tumors, express the cytokine transgene at the metastatic sites, and elicit anti-tumor immune responses, and (2) to determine the safety of IV-administered, genetically-modified endothelial cells.

This report covers the investigation of the safety and therapeutic potential of IV administered hIL-2/MLECs in tumor-bearing mice.

2. BODY

2.1 Specific Aims and Statement of Work

The specific aims of this research are (1) To determine (a) whether IV-injected, interleukin-2 gene-modified murine lung endothelial cells (IL-2/MLECs) can target sites of pulmonary metastases of breast cancer, and (b) how well IL-2/MLECs can express the IL-2 transgene at the metastatic sites; (2) To determine whether the expression of hIL-2 transgene at the local site of pulmonary metastases will induce an anti-tumor immune response. The approved Statement of Work is as follows:-

Task 1: **Months 1-24.**

Determine (a) whether IV-injected, interleukin-2 gene-modified murine lung endothelial cells (IL-2/MLECs) can target sites of pulmonary metastases of breast cancer, and (b) how well IL-2/MLECs can express the IL-2 transgene at the metastatic sites.

a. Mouse lung endothelial cells (MLECs) will be isolated and enriched using FDG-FACS. The cells will be transduced with a retroviral vector containing human IL-2 gene.

b. Efficiency of IL-2/MLEC incorporation at different tumor sites:
   - Co-localization of IL-2/MLEC and tumor in animals: three experiments; 40 animals per

experiment.

c. Determination of toxicity of IV IL-2/MLEC administration:
- Acute toxicity following a single dose of 10^5 IL-2/MLEC administration
- Cumulative toxicity following 3 IV injections of 10^5 IL-2/MLECs spaced 3-4 days apart.
Three experiments; 40 animals per experiment.

d. Optimization of IL-2/MLEC incorporation in tumor sites:
-Tumor-bearing animals will receive three IV injections of IL-2/MLECs closely (3-4 days) or widely (5-7) apart. Expression of IL-2 transgene at the metastatic sites determined by RNA PCR amplification of human IL-2 in discrete individual metastases. Four experiments; 40-50 animals per experiment will be performed.
-Comparison of the relationship between different administration schedules with the number of cells incorporated at sites of tumor metastases will be determined. Two experiments; 40 animals per experiment will be performed.

Task 2: **Months 24-36.**

Determine whether the expression of hIL-2 transgene at the local site of pulmonary metastases site will induce an anti-tumor immune response.

Groups of experimental of experimental and control animals will be sacrificed weekly to monitor hIL-2 expression in the lungs, quantitate metastases, and to assess lung tumor response to IL-2/MLEC treatment. One group of the experimental and control animals will be observed over time for survival. Survivors will receive additional MFP injection of 4T1 cells to determine their ability to reject tumor re-challenge.

2.2 Major Research Accomplishments

Overview

During the past year, we completed the experiments outlined in task 1 and made significant progress in studies outlined in task 2. Specifically, we successfully generated interleukin-2 gene-modified murine lung endothelial cells (IL-2/MLECs) which were used to evaluate the therapeutic potential of intravenously-administered hIL-2/MLECs in mice with lung metastases of 4T1 breast cancer.

Methods

Endothelial cells were harvested from the lungs of BALB/c mice and transduced with a retroviral vector containing the rhIL-2 gene under the transcriptional control of a CMV promoter as previously reported (3). These cells (hIL-2/MLECs) secrete 76 ng (1000 IU)/10^6/24h) of recombinant human interleukin-2 *in vitro*. Tumor-bearing mice were developed by injecting of 10^5 4T1 tumor cell line (derived from a mammary tumor in BALB/c mouse) into the mammary fat pad of syngeneic mice. Two weeks later, at the time when the earliest pulmonary metastases are observed, the primary tumors were completely excised, and mice were randomized into three treatment groups. Three days after surgical excision of the

primary tumor, three sequential tail vein injections of 10^5 hIL-2/MECs or control cells *neo*/GMECs, spaced 72-h apart were administered to mice in groups 1 and 2, respectively. Tumor-bearing mice in group 3 did not receive any GMEC treatment. At weekly intervals, groups of animals were sacrificed, and their lungs removed and examined for the presence and number of tumor foci in order to follow the effect of hIL-2/MECs on the growth of tumor metastases over time. Animals that died or sacrificed when too ill also had their lungs removed and examined.

Results and Discussion

Results

Having established that multiple IV administration of IL-2/MECs can effectively target tumor metastases with adverse effects in animals (see 1999 annual report); we next evaluated the therapeutic utility of this strategy in a spontaneous metastasis model in mice. As shown in Figure 1A, the lungs of untreated tumor-bearing mice and tumor-bearing mice treated with endothelial cells containing the empty vector (LNCX/MECs) had numerous tumor foci. All these mice were dead by day 23 (Figure 2). Their average survival time was 21 days. In contrast, 4T1 tumor-bearing mice treated with the hIL-2/MEC had an average of fifty tumor foci per mouse (Figure 1B), and an average survival time of 40 days (Figure 2). Animals, which received only the hIL-2/MEC without tumor cells, did not develop tumors and remained alive and well.

Apart from the lung tumors, we did not detect hIL-2 gene in any other tissue obtained from the hIL-2/MECs-treated mice. Also, tissues obtained from mice that were treated with endothelial cells containing the vector alone without the hIL-2 gene insert (LNCX/MECs) were hIL-2 gene-negative. Furthermore, we did not observe any tumor in normal mice, which were given the same three IV injections of hIL-2/MECs, suggesting that systemic administration of gene-modified endothelial cells did not promote the formation of tumors in these animals.

Discussion

The results of the present experiments demonstrate that systemic administration of genetically-modified endothelial cells (GMECs) constitutively expressing hIL-2 transgene can mediate the regression of established tumor metastases and prolong the survival of the tumor-bearing animals. The administration of GMECs with empty vector (LNCX/MECs) did not elicit any reduction in tumor burden, suggesting that the tumor regression was mediated by the hIL-2, presumably by stimulation of a local anti-tumor effector cells (4, 5). Of interest, the administration of hIL-2-secreting GMECs did not produce any observable toxicity, such as body weight loss and lethargy, that is frequently reported in animals after systemic administration of IL-2 therapy (5). In contrast to these findings, others have reported that IV-administered NIH3T3 (6) or B16F10 melanoma cells (7) transduced to express IL-2 alone were unable to significantly reduce the rate of tumor growth. Since only a fraction of IV-administered cells could migrate to the site of tumors, the difference in these results may be related, in part, to the ability of IL-2/GMECs to divide in response to the angiogenic factors at the tumor sites (8). Consequently, GMECs, but not the IL-2/NIH3T3 or lethally irradiated IL-2/B16F10 used in these other studies, are able to grow and produce the levels of the therapeutic gene product that is sufficient to elicit an anti-tumor effect.

In summary, three IV injections of 10^5 hIL-2/MECs, spaced at 3 days apart, abrogated established lung metastases of breast cancer and prolonged the survival of the animals. These results suggest that targeting tumor metastases using endothelial cells that have been genetically modified to express therapeutic transgene(s) is a potentially safe and effective strategy to treat metastatic diseases.

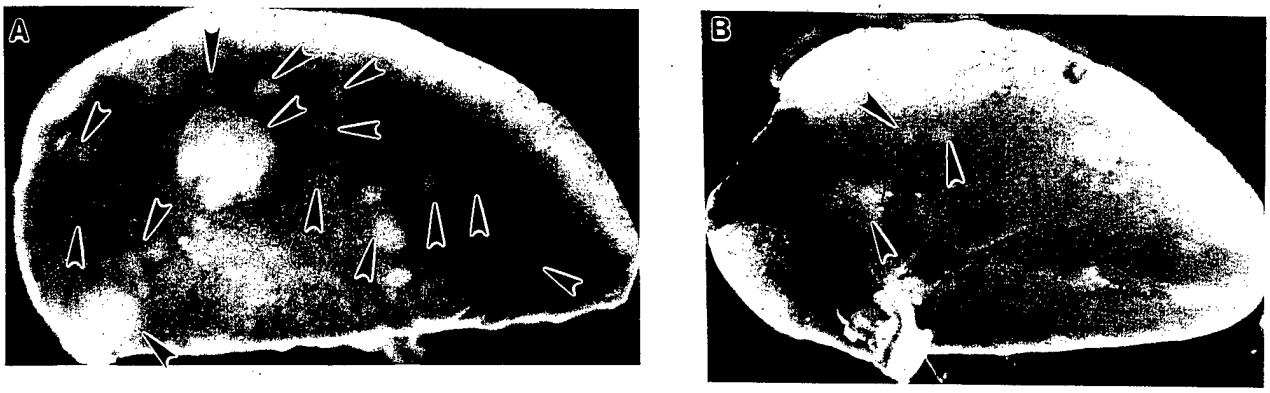

Figure 1: Macroscpoic appearance of lungs from untreated breast tumor-bearing BALB/c mice (Controls, A) and tumor-bearing mice treated with IL-2 transgene-expressing endothelial cells (B). BALB/c mice (5 or more per group) were injected with 1 x 10^5 4T1 cell line into the mammary fat pad. Two weeks later, a time period when pulmonary metastasis is fully established, the primary tumor was excised to prevent further metastatic dissemination. Three days after primary tumor excision, the mice received the first of three intravenous injections of 1 x 10^5 IL-2/MLECs or LNCX/MLECs (control), spaced at three days apart. The mice were sacrificed three to four weeks post treatment. Note the presence of numerous metastatic foci (arrows heads) in the lung from the untreated mouse, (A), while the lung from treated mice, (B), contain fewer metastatic foci (arrow heads). Magnification, x 25.

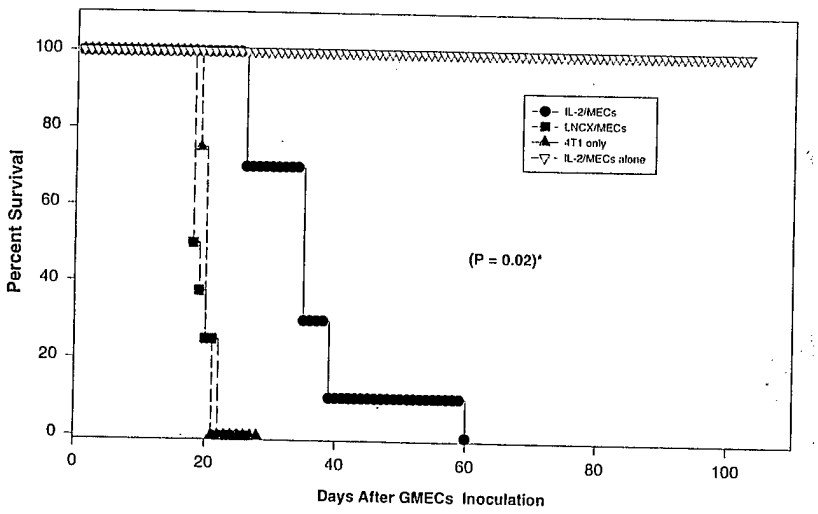

Figure 2. Survival of 4T1 breast cancer-bearing BALB/c mice treated with IL-2 transgene-expressing endothelial cells. BALB/c mice (8 or more per group) were injected with 1 x 10^5 4T1 breast tumor cells into the Mammary Fat Pad. Two weeks later, when lung mestastases were fully established, the primary tumors were completely excised. Three days after the surgical excision of primary tumor, a group of mice were given the first of three intravenous injections of 1 x 10^5 IL-2/MECs, each spaced at three days apart. The average survival times (days) after the injection of 4T1 cells: Untreated, 21.0 days; Vector (LNCX/MEC)-treated, 22 days; IL-2/MEC-treated, 40.0 days; and IL-2/MEC-treated non-tumor-bearing mice, no death. P value relative to untreated tumor-bearing mice <0.02.

2.3 Plans for the Future

In the coming year, we plan to complete all the remaining experiments under this research. Specifically, further experiments will be performed to determine whether IV-injected hIL-2/MLECs can lodge within an inactive or a physiologically active site in mice. We also plan to determine a) the nature, level, and duration of anti-tumor immune response that is induced at the local tumor site; and b) the ability of microvascular endothelial cells (MECs) expressing interleukin-12 (IL-12) transgene or herpes simplex thymidine kinase (HSV-TK) gene to inhibit the growth of established breast cancer metastases mice.

3. Key Research Accomplishments

We have:

1) Isolated pure population of lung endothelial cells from BALB/c mice. The cells have been transduced with a retroviral vector containing human IL-2 gene and high expressing clones have been isolated and fully characterized.
b) Determined the efficiency of hIL-2/MLEC incorporation into sites of breast cancer metastasis.
c) Optimized hIL-2/MLEC incorporation into sites of pulmonary metastasis of breast cancer.
d) Determined acute and cumulative toxicity of IV-administered hIL-2/MLECs.
e) Established that the expression of hIL-2 transgene at the local site of pulmonary metastases site can induce an anti-tumor immune response *in vivo*.

4. Reportable Outcomes

The following two abstracts of this research have been presented at national conferences.
(a) "Interleukin-2 gene-modified endothelial cell targeting of breast cancer metastases in mice" was presented at the U.S. Department of Defense (DOD) Breast Cancer Research Program (BCRP) Era of Hope meeting at Hilton Atlanta and Towers, Atlanta, Georgia, June 2000. (Appendix 1).
(b) "Interleukin-2 gene-modified endothelial cell treatment of metastatic breast cancer in mice" was presented at the American Association for Cancer Research's special conference on Gene regulation and Cancer, held at Hot Springs, Virginia, in October 1999 (appendix 2).

5. Conclusions

These results demonstrate that: - 1) genetically-modified endothelial cells can express interleukin-2 transgene at the local site of breast cancer metastasis, 2) three sequential IV injections of 10^5 hIL-2/MLECs, given at 72h intervals, can abrogate lung metastases of breast tumor and prolong the survival of the animal, and 3) multiple IV injections of 10^5 hIL-2/MLECs, spaced 72h apart is safe in mice. These results suggest that systemic administration of genetically-modified endothelial cells is an effective and safe therapeutic strategy for the treatment of cancer metastases. The approach may be particularly useful for targeting recombinant therapeutic molecules to sites of macrometastases throughout the body.

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Appendices:

Two abstracts presented at national meetings.

(a) Ojeifo JO, Herscowitz HB, Zwiebel JA. Interleukin-2 gene-modified endothelial cell targeting of breast cancer metastases mice. Proceedings of the U.S. Department of Defense (DOD) Breast Cancer Research Program (BCRP) Era of Hope meeting at Hilton Atlanta and Towers, Atlanta, Georgia, June, 2000. (abstr. # AA-15).

(b) Ojeifo JO, Vezza P, Kallakhury B, Lippman ME. Interleukin-2 gene-modified endothelial cell treatment of metastatic breast cancer in mice. Proceedings of Gene Regulation and Cancer, American Association For Cancer Research's special conference, Hot Springs, Virginia, October 1999 (abstr. # B-14).

INTERLEUKIN-2 GENE-MODIFIED ENDOTHELIAL CELL TARGETING OF BREAST CANCER METASTASES IN MICE.

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Targeting of tumor metastases using genetically modified cells is an attractive strategy for effective gene therapy of invasive breast cancer. Previous studies in our laboratory have shown that *LacZ* gene-expressing endothelial cells (GMECs) administered intravenously (IV) can become incorporated into sites of active angiogenesis. The purposes of the present study were (a) to determine whether interleukin-2 gene-modified endothelial cells (IL-2/GMECs) administered intravenously could target sites of breast cancer metastases, and (b) to define the optimal dose and toxicity of IL-2/GMEC administration.

Two weeks after the establishment of breast cancer metastases in the lungs of BALB/C mice, animals were treated with IV injections of 10^5 to 10^7 IL-2/GMECs via tail vein. At varying intervals after IL-2/GMEC administration, lungs and other tissues were harvested and examined for presence and expression of hIL-2 gene by the polymerase chain reaction (PCR) and reverse transcriptase (RT)-PCR techniques, respectively.

In mice treated with a single injection of 10^5 hIL-2/GMECs, only 2, 5, and 2% of the lung metastases were positive for hIL-2 on days 7, 14, and 21, respectively. Animals that received single or multiple injections of 10^6 or 10^7 of hIL-2/GMECs died from toxicity. In contrast, 80, 90, and 30% of lung metastases recovered from mice which had three sequential IV injections of 10^5 hIL-2/GMECs, at 3-day intervals, were positive for hIL-2 on days 7, 14, and 21, respectively with no deleterious effects in the animals. Neither the presence nor the expression of hIL-2 gene was detected in any other tissues of the hIL-2/GMEC-treated mice, or in untreated, tumor-bearing mice. These results suggest that IV-administered, IL-2/GMECs can target tumor metastases in mice and continue to express exogenous IL-2 gene at the tumor sites. This approach may be useful for targeting recombinant therapeutic molecules to sites of metastatic deposits throughout the body.

This work was supported by the U.S. Army Medical Research and Materiel Command Grant # DAMD17-98-1-8094.

Interleukin-2 gene-modified endothelial cell treatment of metastatic breast cancer in mice

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Recurrence and metastatic dissemination of breast cancers account for significant morbidity and mortality rates in women. Efficient means of treating this subset of patients remain elusive. The goal of this study is to determine whether intravenous (IV) injection of human Interleukin-2 gene-modified murine microvascular endothelial cells (hIL-2/MECs) can abrogate breast cancer metastases and prolonged the survival of the tumor-bearing mice.

This study involves the injection of 10^5 cells from a 4T1 tumor cell line (derived from a mammary tumor in BALB/c mouse) into the mammary fat pad of BALB/C mice. Two weeks later, at the time when the earliest pulmonary metastases are observed, the primary tumors were excised, and three IV injections of hIL-2/MECs (3- day intervals) were administered. Following the death or sacrifice of the animals, their lungs were removed and examined for the presence and number of tumor foci.

Untreated tumor-bearing mice and tumor-bearing mice treated with neomycin gene-modified endothelial cells had tumor foci that were too numerous to count. These animals all died by day 23. Their average survival time was 20 days. In contrast, 4T1 tumor-bearing mice treated with the hIL-2/MEC had an average of 50 tumor foci per mouse and an average survival time of 40 days. Animals that received only the hIL-2/MEC without tumor cells did not develop tumors and remained alive and well. These results suggest that IV- administered, interleukin-2 gene-modified endothelial cells can inhibit the growth of established metastases of breast cancer and prolong the survival of tumor-bearing animals.

This work was supported in part by the U.S. Army Medical Research and Materiel Command Grant # DAMD17-98-1-8094.